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Short Communication

Genome sequence of *Rhizomucor pusillus* FCH 5.7, a thermophilic zygomycete involved in plant biomass degradation harbouring putative GH9 endoglucanases

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ABSTRACT

We report here the annotated draft genome sequence of the thermophilic zygomycete *Rhizomucor pusillus* strain FCH 5.7, isolated from compost soil in Vietnam. The genome assembly contains 25.59 Mb with an overall GC content of 44.95%, and comprises 10,898 protein coding genes. Genes encoding putative cellulose-, xylan- and chitin-degrading proteins were identified, including two putative endoglucanases (EC 3.2.1.4) from glycoside hydrolase family 9, which have so far been mostly assigned to bacteria and plants.

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1. Introduction

Fast growth and rapid spore production mark the thermophilic zygomycete *Rhizomucor pusillus*, a member of the Mucorales, as a mycoflora pioneer species in compost [1,2]. A number of its encoded enzymes have been produced and characterised, such as polygalacturonase, glycoamylase, phytase and glucanase [3–6], and particularly the high temperature optima of *R. pusillus* enzymes of around 60–70 °C [5,6] make the fungus an interesting source of biocatalysts. A lipase of a close relative, *Rhizomucor miehei*, is commercially available and used industrially (Palatase[®], Novozymes) [7]. Therefore, *R. pusillus* represents a promising resource for the exploration of useful enzymes. The *R. pusillus* strain FCH 5.7 described in this study was isolated from 3 month old compost in Ly Nhan, Ha Nam province, Vietnam (geographical location 20°33′06.2″ N, 106°04′11.8″ E). Genome sequencing was

performed to enable a systematic exploration, particularly of the strain's carbohydrate-active enzymes (CAZymes) [8], as well as to set the basis for investigating its growth strategies at higher temperatures.

2. Materials & methods

After isolation, *R. pusillus* FCH 5.7 was cultivated on potato dextrose agar (PDA) at 50 °C and the species identified by internal transcribed spacer (ITS) sequencing, as described previously [9]. For whole genome sequencing, 5-day-old mycelium from PDA plates was used for the inoculation of basal liquid medium (4 g L⁻¹ KH₂PO₄, 13.6 g L⁻¹ (NH₄)₂SO₄, 0.8 g L⁻¹ CaCl₂·2H₂O, 0.6 g L⁻¹ MgSO₄·7H₂O, 6 g L⁻¹ Bacto peptone, 10 mg L⁻¹ FeSO₄·7H₂O, 3.2 mg L⁻¹ MnSO₄·H₂O, 2.8 mg L⁻¹ ZnSO₄·7H₂O, 4 mg L⁻¹ CoCl₂·6H₂O, 200 mL L⁻¹ Tween 80, pH 5.8) containing 20 g L⁻¹ glucose as carbon source. Cultivation was carried out in 125 mL liquid medium in baffled Erlenmeyer flasks (500 mL; 50 °C, 48 h, shaking at 250 rpm). The mycelium was harvested after two days of cultivation and DNA was extracted with cetyltrimethyl ammonium bromide (CTAB) buffer (2% CTAB, 100 mM TrisHCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl) and purified from the supernatant by a combination of phenol-chloroform extraction and isopropanol precipitation and DNeasy Plant Mini Kit (Qiagen), as described

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previously [9]. Quality of the purified DNA was verified by agarose gel electrophoresis, Nanodrop (Thermo Scientific) and Qubit (Life Technologies) before genome sequencing. RNA was extracted from cultures growing in basal liquid medium, supplemented with 1% glucose, wheat bran or beechwood xylan, respectively, after 4 h and 48 h, using TRIzol (Invitrogen) and chloroform, according to the manufacturer's instructions. Enzymatic activities (xylanase, endoglucanase) were determined in supernatant samples from the same cultivations through measurement of produced reducing ends, using the 3,5-dinitrosalicylic acid DNS method [10].

For genome sequencing, the NEBNext Ultra DNA Library Prep kit for Illumina (New England Biolabs) was used to process the DNA samples according to the manufacturer's protocol. After fragmentation of DNA with a Covaris ultrasonicator (Thermo Scientific), quality and yield were measured with a Bioanalyzer (Agilent Technologies). Clustering and DNA sequencing with the Illumina cBot and HiSeq 2500 were performed with 8.0pM of DNA, standard Illumina primers and HiSeq control software HCS v2.2.58. Image analysis, base calling, and quality check were done with the Illumina data analysis pipeline RTA v1.18.64 and Bcl2fastq v1.8.4. The GenomeScan in-house tool FASTQFilter v2.05 was used for adapter trimming and quality filtering of the 250 bp paired-end reads. The short-read genome assembler Abyss v1.3.7 [11] with a k-mer length of 64 was used for assembly. Scaffolds shorter than 500 bp were removed.

For RNA sequencing, total RNA was further prepared with the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) with oligo-dT magnetic beads for mRNA enrichment. Sequencing of the resulting cDNA (16 pM) was done with an Illumina cBot and HiSeq 2500 instrument. The newly assembled genome was used as a reference to map the mRNA-Seq reads using the packages Tophat (v2.0.14, Linux_x86_64) and Bowtie (v2-2.1.0) with a default mismatch rate of 2%. Gene finding was performed combining two approaches. The HMM-based algorithm Glimmer v3.02 [12] was trained using the genome of *Mucor circinelloides* (downloaded from the Joint Genome Institute (JGI), <http://genome.jgi.doe.gov/>). In addition, an evidence-based method of gene finding was performed using mapped mRNA-Seq reads and the software tool CodingQuarry [13]. Combining the two methods, gene models for 10,898 genes were obtained. For annotation, a BLASTp search (version 2.2.28+) (<http://blast.ncbi.nlm.nih.gov>) was performed on the UniprotKB/SwissProt database with default parameters. For comparison to other species, publicly accessible genomes were downloaded from JGI Mycocosm (<https://genome.jgi.doe.gov/programs/fungi/index.jsf>, Supplemental File 1).

The CAZyme contents of *R. pusillus* FCH 5.7 and the fungal species included for comparison were determined by identifying genes containing CAZyme domains using the dbCAN2 meta server (cys.bios.niu.edu/dbCAN2). Only CAZyme domains predicted by at least two of the three algorithms (DIAMOND, HMMER and Hotpep) employed by dbCAN2 were kept. It should be noted that the stringency of this approach led to slightly different numbers of identified CAZymes than has previously been reported for the respective species.

3. Results & discussion

The 25.59 Mb genome of *R. pusillus* FCH 5.7 was obtained with 294-fold coverage (Table 1). The whole genome assembly has been submitted to the European Nucleotide Archive and deposited at DDBJ/EMBL/GenBank under the assembly accession number FWWN00000000.

The analysis of CAZyme-encoding genes showed that *R. pusillus* FCH 5.7 possesses a similar number of total CAZymes as other zygomycetes, such as *Hesseltinella vesiculosa* and *Phycomyces*

Table 1

Genome features of *R. pusillus* FCH 5.7.

Genome assembly size (Mb)	25.59
Genome coverage (x times)	294
Number of scaffolds (≥500 bp)	618
Contig N50 (bp)	102,680
Number of scaffolds > N50	77
Max scaffold size (bp)	534,899
Number of protein coding genes	10,898
Mean gene length (bp)	1,359
GC content, overall (%)	44.95
GC content, coding (%)	48.43

blakesleeanus, but considerably fewer than *Lichtheimia corymbifera* and *Rhizopus oryzae* (Table 2), which could be due to the reported gene expansion and genome duplication in *L. corymbifera* and *R. oryzae*, respectively [14,15]. The difference was particularly obvious for carbohydrate esterases (CE) and glycoside transferases (GT), two CAZy enzyme classes that are known to be highly abundant in *R. oryzae* [16]. Compared to basidiomycetes, zygomycetes generally have higher numbers of GTs, and this was also true for *R. pusillus*, which harbours 77 CAZy domains across 27 GT families in its genome. The number of auxiliary activity (AA) enzymes was relatively small in the investigated zygomycetes, and *R. pusillus* FCH 5.7 was found to only possess members of AA families 1, 5 and 6, as well as one member in family 12. None of the zygomycetes used for comparison contained AA9 lytic polysaccharide monoxygenases (LPMOs), which are abundant in ascomycetes and basidiomycetes and involved in cellulose degradation (Supplemental File 1; [17]).

Growth on plates containing basal medium and 1% beechwood xylan was very poor, and *R. pusillus* FCH 5.7 grew much better on mono- and disaccharides (glucose, galactose, mannose, cellobiose), as well as starch (Fig. 1A). *R. pusillus* FCH 5.7 was not able to grow efficiently on cellulose (Avicel), which indicates a lack of endoglucanases and/or cellobiohydrolases to break down this polymer. Indeed, no putative GH6 or GH7 cellobiohydrolases could be found in the *R. pusillus* FCH 5.7 genome. The ability to degrade chitin was indicated by the presence of many chitinases (GH18) and chitin deacetylases (CE4) in the genome, and was corroborated by the ability of *R. pusillus* FCH 5.7 to grow on chitosan (Fig. 1A, Supplemental File 1). In contrast to a previous study [4], *R. pusillus* FCH 5.7 grew quite poorly on pectin, and many genes typically involved in pectin degradation, such as pectin and pectate lyases (PL1, 3, 4), were found missing in its genome. We did, however, detect sequences of three GH28s that encode putative polygalacturonases. Similar to *R. miehei* [18], a large number of lipases was found in the genome of *R. pusillus* FCH 5.7, with in total 80 putative lipases and phospholipases.

Despite the poor growth on plates containing Avicel or xylan, xylanase and low levels of endoglucanase (CMCase) activities were

Table 2

Comparison of the numbers of CAZymes in *R. pusillus* FCH 5.7 with those in other fungi.

		total	AA	CBM	GH	CE	PL	GT
ZYG	<i>Rhizomucor pusillus</i>	184	11	1	81	12	2	77
	<i>Absidia repens</i>	236	11	0	78	19	2	126
	<i>Hesseltinella vesiculosa</i>	183	11	1	67	13	1	90
	<i>Lichtheimia corymbifera</i>	261	11	2	93	28	1	126
	<i>Mucor circinelloides</i>	221	10	2	74	23	2	110
	<i>Phycomyces blakesleeanus</i>	195	9	2	66	19	3	96
	<i>Rhizopus microsporus</i>	201	11	3	72	17	3	95
	<i>Rhizopus oryzae</i>	263	14	4	105	24	3	113
	<i>Syncephalastrum racemosum</i>	239	11	3	96	24	2	103
ASC	<i>Thielavia terrestris</i>	405	65	28	202	23	4	83
BAS	<i>Schizophyllum commune</i>	331	56	6	175	19	15	60

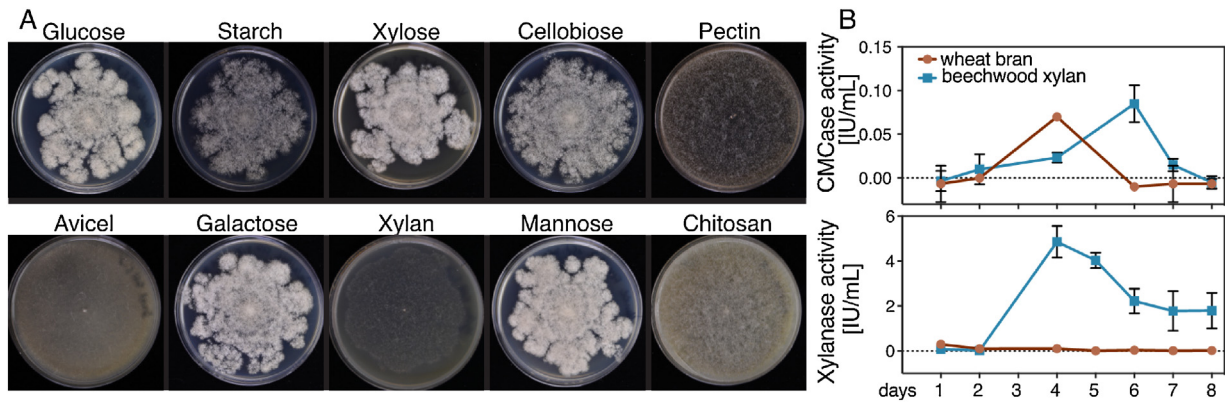


Fig. 1. Growth on plates containing 0.1 g L^{-1} of a single carbon source (A), and enzyme assays evaluating endoglucanase (CMCase) and xylanase activities (B). For plates, fungus was grown at 50°C for 3 days. For enzyme assays, fungus was cultivated on wheat bran (red) or beechwood xylan (blue) over the course of 8 days.

detected in liquid cultures of *R. pusillus* FCH 5.7 grown on wheat bran or beechwood xylan as carbon sources (Fig. 1A). Cultivation on wheat bran and beechwood xylan resulted in almost identical levels of CMCase activity. Xylanase activity, on the other hand, was much higher during growth on xylan compared to growth on wheat bran. This indicates the presence of xylanases that are induced more by hardwood glucuronoxylans, as found in beechwood, rather than the grass-specific arabinoxylans contained in wheat bran.

Several putative endoglucanases from family GH45 were detected in the genome, which could explain the low enzyme activity observed on CMC (Fig. 1B). Interestingly, *R. pusillus* FCH 5.7 also encodes two GH9 members, which is a family containing endoglucanases typically found in bacteria, plants and occasionally animals [19,20], but completely absent in ascomycetes fungi. We compared the genomes of a selection of fungi to determine how widespread the occurrence of GH9 genes is. Many of the basidiomycetes in our selection were shown to encode a single putative GH9 protein, while up to four were detected in zygomycetes (Fig. 2). Zygomycete GH9s are, to our knowledge, completely unexplored proteins and the function of fungal GH9s and the importance for, for instance, growth on polysaccharides is unclear and has not been described yet. One study indicated an involvement in degradation of crystalline cellulose, as a putative

GH9 gene was found to be upregulated in the basidiomycete *P. chrysosporium* grown on cellulose [21], but conclusive functional evidence is still missing.

The lifestyle and habitat, and therefore the repertoire of CAZymes of zygomycetes is quite different to ascomycetes and basidiomycetes. The enzyme system of *R. pusillus* FCH 5.7 seems to be better suited to consume easily accessible, simple sugars found in plant biomass [22] as well as lipid-containing compounds. As seen in the growth studies (Fig. 1A) and also shown for *R. oryzae*, zygomycetes are in general unable to degrade complex plant cell wall polysaccharides [16]. The lack of genes encoding enzymes of the CAZy families GH6, GH7 and AA9 in the *R. pusillus* genome further supports this statement. However, the low endoglucanase and xylanase activities detected in *R. pusillus* FCH 5.7 culture supernatants, as well as the presence of putative xylanases and endoglucanases (GH9, GH45) in the genome indicate that this fungus may be able to degrade oligo- and/or polysaccharide to a certain extent.

3.1. Nucleotide sequence accession number(s)

The *R. pusillus* FCH 5.7 whole genome sequence has been submitted to GenBank and deposited at DDBJ/EMBL/GenBank under the accession No. FWWN00000000.

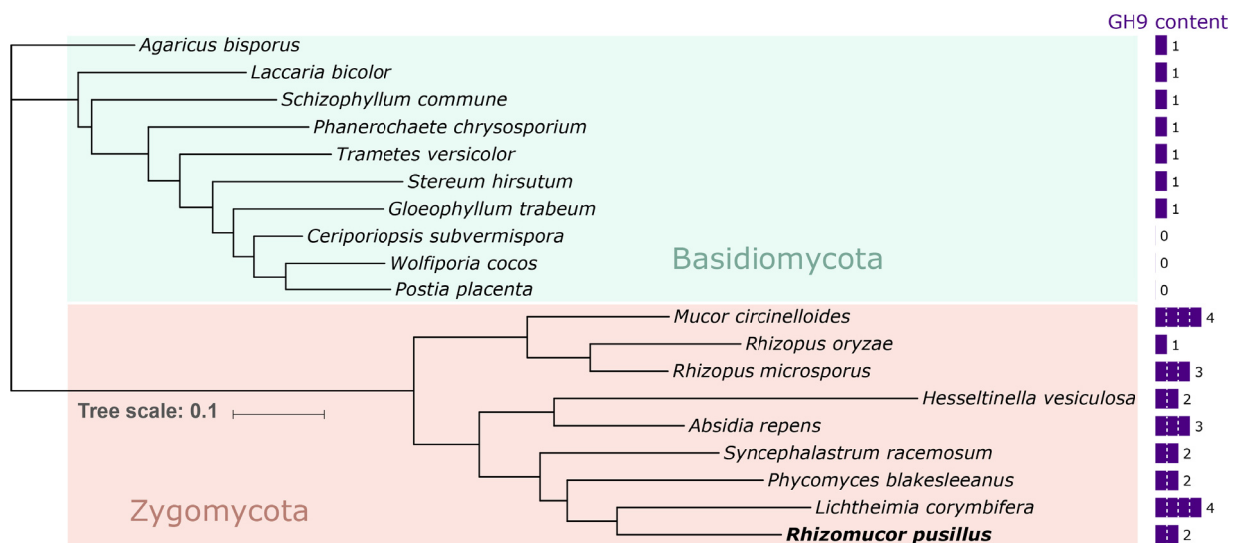


Fig. 2. Maximum-likelihood phylogenetic tree depicting the relationships among selected fungal species and the GH9 content of their respective genomes. The tree was constructed based on protein sequences of GT33 proteins, which are present as a single copy in each of the selected species, in MEGA7 [23] and was annotated with iTOL [24].

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2018.e00279>.

References

- [1] T. Satyanarayana, J. Littlechild, Y. Kawarabayasi, Thermophilic Microbes in Environmental and Industrial Biotechnology, (2013), doi:<http://dx.doi.org/10.1007/978-94-007-5899-5>.
- [2] I. Morgenstern, J. Powlowski, N. Ishmael, C. Darmond, S. Marquetteau, M.C. Moisan, G. Quenneville, A. Tsang, A molecular phylogeny of thermophilic fungi, Fungal Biol. 116 (2012) 489–502, doi:<http://dx.doi.org/10.1016/j.funbio.2012.01.010>.
- [3] L. Viikari, M. Alapuranen, T. Puranen, J. Vehmaanperä, M. Siika-Aho, Thermostable enzymes in lignocellulose hydrolysis, Adv. Biochem. Eng. Biotechnol. 108 (2007) 121–145, doi:http://dx.doi.org/10.1007/10_2007_065.
- [4] M.A. Siddiqui, V. Pande, M. Arif, Production, purification, and characterization of polygalacturonase from rhizomucor pusillus isolated from Decomposting Orange Peels, Enzyme Res. 2012 (2012) 138634, doi:<http://dx.doi.org/10.1155/2012/138634>.
- [5] B.S. Chadha, G. Harmeet, M. Mandeep, H.S. Saini, N. Singh, Phytase production by the thermophilic fungus *Rhizomucor pusillus*, World J. Microbiol. Biotechnol. 20 (2004) 105–109, doi:<http://dx.doi.org/10.1023/B:WIBL.0000013319.13348.0a>.
- [6] Z. He, L. Zhang, Y. Mao, J. Gu, Q. Pan, S. Zhou, B. Gao, D. Wei, Cloning of a novel thermostable glucoamylase from thermophilic fungus *Rhizomucor pusillus* and high-level co-expression with α -amylase in *Pichia pastoris*, BMC Biotechnol. 14 (2014) 1–10, doi:<http://dx.doi.org/10.1186/s12896-014-0114-8>.
- [7] R.C. Rodrigues, R. Fernandez-Lafuente, Lipase from *Rhizomucor miehei* as an industrial biocatalyst in chemical process, J. Mol. Catal., B Enzym. 64 (2010) 1–22, doi:<http://dx.doi.org/10.1016/j.molcatb.2010.02.003>.
- [8] V. Lombard, H. Golaconda Ramulu, E. Drula, P.M. Coutinho, B. Henrissat, The carbohydrate-active enzymes database (CAZy) in 2013, Nucleic Acids Res. 42 (2014) D490–D495, doi:<http://dx.doi.org/10.1093/nar/gkt1178>.
- [9] S. Hüttner, T.T. Nguyen, Z. Granchi, T. Chin-A-Woeng, D. Ahrén, J. Larsbrink, V.N. Thanh, L. Olsson, Combined genome and transcriptome sequencing to investigate the plant cell wall degrading enzyme system in the thermophilic fungus *Malbranchea cinnamomea*, Biotechnol. Biofuels 10 (2017), doi:<http://dx.doi.org/10.1186/s13068-017-0956-0>.
- [10] Z. Xiao, R. Storms, A. Tsang, Microplate-based carboxymethylcellulose assay for endoglucanase activity, Anal. Biochem. 342 (2005) 176–178, doi:<http://dx.doi.org/10.1016/j.ab.2005.01.052>.
- [11] J.T. Simpson, K. Wong, S.D. Jackman, J.T. Simpson, K. Wong, S.D. Jackman, J.E. Schein, S.J.M. Jones, ABYSS : a parallel assembler for short read sequence data, Genome Res. 19 (2009) 1117–1123, doi:<http://dx.doi.org/10.1101/gr.089532.108>.
- [12] W.H. Majoros, M. Pertea, S.L. Salzberg, TigrScan and GlimmerHMM: two open source ab initio eukaryotic gene-finders, Bioinformatics 20 (2004) 2878–2879, doi:<http://dx.doi.org/10.1093/bioinformatics/bth315>.
- [13] A.C. Testa, J.K. Hane, S.R. Ellwood, R.P. Oliver, CodingQuarry: highly accurate hidden Markov model gene prediction in fungal genomes using RNA-seq transcripts, BMC Genomics 16 (170) (2015), doi:<http://dx.doi.org/10.1186/s12864-015-1344-4>.
- [14] J. Linde, V. Schwartz, U. Binder, K. Voigt, F. Horn, *De novo* whole-genome sequence and genome annotation of *Lichtheimia ramosa*, Genome Announc. 2 (2014) e0088–14, doi:<http://dx.doi.org/10.1128/genomeA.00888-14>. Copyright.
- [15] L.J. Ma, A.S. Ibrahim, C. Skory, M.G. Grabherr, G. Burger, M. Butler, M. Elias, A. Idnurm, B.F. Lang, T. Sone, A. Abe, S.E. Calvo, L.M. Corrochano, R. Engels, J. Fu, W. Hansberg, J.M. Kim, C.D. Kodira, M.J. Koehrsen, B. Liu, D. Miranda-Saavedra, S. O'Leary, L. Ortiz-Castellanos, R. Poulter, J. Rodriguez-Romero, J. Ruiz-Herrera, Y. Q. Shen, Q. Zeng, J. Galagan, B.W. Birren, C.A. Cuomo, B.L. Wickes, Genomic analysis of the basal lineage fungus *Rhizopus oryzae* reveals a whole-genome duplication, PLoS Genet. 5 (2009), doi:<http://dx.doi.org/10.1371/journal.pgen.1000549>.
- [16] E. Battaglia, I. Benoit, J. van den Brink, A. Wiebenga, P.M. Coutinho, B. Henrissat, R.P. de Vries, Carbohydrate-active enzymes from the zygomycete fungus *Rhizopus oryzae*: a highly specialized approach to carbohydrate degradation depicted at genome level, BMC Genomics 12 (2011) 38, doi:<http://dx.doi.org/10.1186/1471-2164-12-38>.
- [17] A.V. Monclaro, E.X.F. Filho, Fungal lytic polysaccharide monooxygenases from family AA9: recent developments and application in lignocellulose breakdown, Int. J. Biol. Macromol. 102 (2017) 771–778, doi:<http://dx.doi.org/10.1016/j.ijbiomac.2017.04.077>.
- [18] P. Zhou, G. Zhang, S. Chen, Z. Jiang, Y. Tang, B. Henrissat, Q. Yan, S. Yang, C.-F. Chen, B. Zhang, Z. Du, Genome sequence and transcriptome analyses of the thermophilic zygomycete fungus *Rhizomucor miehei*, BMC Genomics 15 (294) (2014), doi:<http://dx.doi.org/10.1186/1471-2164-15-294>.
- [19] J. Ni, M. Takehara, H. Watanabe, Identification of activity related amino acid mutations of a GH9 termite cellulase, Bioresour. Technol. 101 (2010) 6438–6443, doi:<http://dx.doi.org/10.1016/j.biortech.2010.03.045>.
- [20] A. Davison, M. Blaxter, Ancient origin of glycosyl hydrolase family 9 cellulase genes, Mol. Biol. Evol. 22 (2005) 1273–1284, doi:<http://dx.doi.org/10.1093/molbev/msi107>.
- [21] A. Vanden Wymelenberg, S. Denman, D. Dietrich, J. Bassett, X. Yu, R. Atalla, P. Predki, U. Rudsander, T.T. Teeri, D. Cullen, Transcript analysis of genes encoding a family 61 endoglucanase and a putative membrane-anchored family 9 glycosyl hydrolase from *Phanerochaete chrysosporium*, Appl. Environ. Microbiol. 68 (2002) 5765–5768, doi:<http://dx.doi.org/10.1128/AEM.68.11.5765-5768.2002>.
- [22] M. Richardson, The ecology of the zygomycetes and its impact on environmental exposure, Clin. Microbiol. Infect. 15 (2009) 2–9, doi:<http://dx.doi.org/10.1111/j.1469-0691.2009.02972.x>.
- [23] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. (2016), doi:<http://dx.doi.org/10.1093/molbev/msw054>.
- [24] I. Letunic, P. Bork, Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees, Nucleic Acids Res. 44 (2016) W242–W245, doi:<http://dx.doi.org/10.1093/nar/gkw290>.